

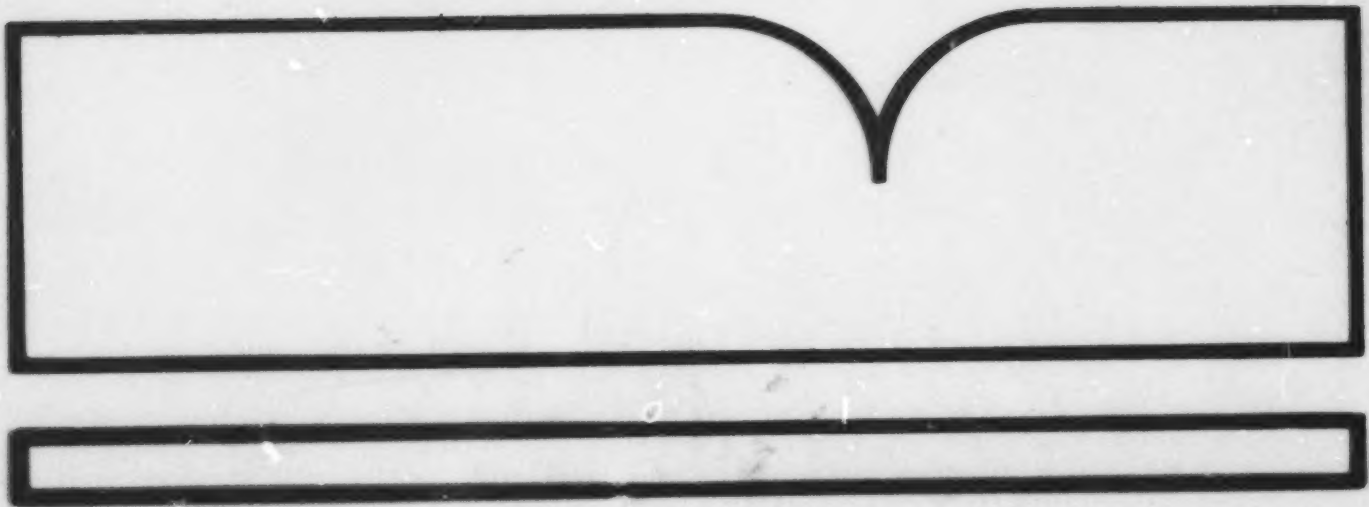
Structure and Organization of Methane Genes

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Structure and Organization of Methane Genes

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1. Abstract

Methanogenesis is a major pathway in the global cycling of carbon. Anaerobic biodegradation is used by man both for detoxification and disposal of wastes and for conversion of biomass to the readily combustible fuel, methane. The microorganisms that synthesize methane, the methanogens, are anaerobic archaeobacteria; however, we have shown that methanogen genes closely resemble eubacterial genes and that they can be functionally expressed in eubacterial cells. The possibility therefore exists to use recombinant DNA procedures to investigate and dissect the regulation and biochemistry of methanogenesis. This paper analyses currently available biochemical information to identify enzymes essential for methanogenesis; enzymes that could therefore be considered to be encoded by methane genes.

2. Introduction

What are methane genes? Answering this question is clearly of central importance for planning genetics and gene cloning experiments to investigate methanogenesis. Methanogens oxidize the simple substrates hydrogen, formate, methanol, methylamines and acetate to gain energy. In the process they reduce methyl groups to methane. The methyl groups may be obtained from methanol, methylamines or acetate or may result from sequential reduction of carbon dioxide via the formyl-, methenyl- and methylene levels of oxidation (1-5). The problem is defining which biochemical reactions are essential for methanogenesis so that genes encoding enzymes that catalyse these reactions can be targeted

for mutagenesis, cloning and *in vitro* manipulation. Methanogenesis appears to be an essential activity for methanogens. Methanogens with alternative life styles have not been found so that it must be assumed that a null mutation in a methane gene in a known species would be lethal. Facultative methanogens may exist, possibly with an energy-generating system based on sulphur metabolism (6); however, until such an organism is available only mutants with conditionally lethal mutations in methane genes could be isolated in species that reduce carbon dioxide or formate to methane. In the metabolically more versatile *Methanosarcina* species it may be possible to isolate mutants incapable of methanogenesis from one substrate, e.g. methylamine, but which are still able to grow by generating methane from other substrates, e.g. methanol or acetate.

Several groups have attempted to isolate mutants and evaluate transformation and conjugation protocols for methanogens. The results to date have, however, been somewhat disappointing. A few antibiotic resistant (7-9) and auxotrophic (10,11) mutants have been described, but unfortunately most of the antibiotic-resistant mutants appear to be resistant because of changes in their cell permeabilities (9). There is, as yet, no native resistance-conferring methanogen plasmid or methanogen transducing phage, although some progress has been made in identifying and reconstructing cryptic plasmids potentially of use as cloning/shuttle vectors (12). There is only one published report of a low-frequency transformation system (11).

In the absence of methane mutants and a convenient genetic exchange system the only current practical approach to studying methane genes is to use gene cloning and analysis in a surrogate host cell system. The remainder of this paper identifies biochemical pathways and reactions catalysed by enzymes that could be considered to be encoded by 'methane genes'. As such, these represent candidate genes for cloning and analysis using recombinant DNA techniques. A frequent post-seminar question is: 'Do you intend to construct an *E. coli* that makes methane?' The difficulty and unlikelihood of reaching such a goal, regardless of its desirability, should become apparent.

3. Overview of the Components of Methanogenesis

The precursors for methanogenesis are electrons (reducing equivalents) and methyl groups (1-3). Methane genes can therefore be defined as genes encoding enzymes that facilitate the supply and subsequent metabolism of these precursors to methane. The activities of many of these enzymes require the participation of cofactors found only in methanogens (2,3). The enzymes that catalyse the biosyntheses of these cofactors must therefore also be considered to be encoded by methane genes until it can be shown that alternative cofactors can substitute for the methanogen-specific cofactors. The metabolic pathways catalysed by enzymes considered to be encoded by methane genes are shown in Figure 1.

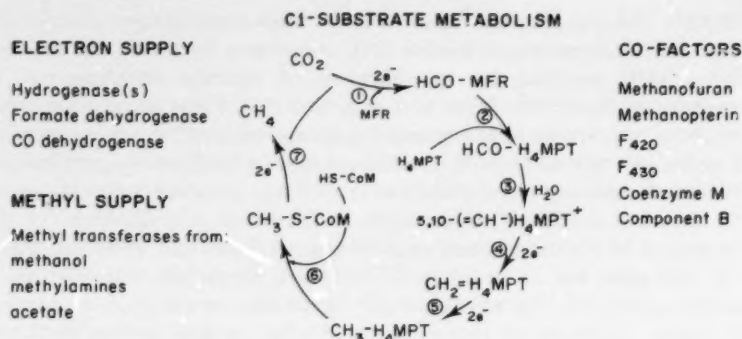


Figure 1. An overview of metabolism catalysed by enzymes considered to be encoded by methane genes. (Reproduced from Jones WP, Nagle DP Jr, Whitman WB. *Microbiol Rev* 1987; 51: 135-177, by permission of the American Society for Microbiology.)

The current status and future opportunities for identifying and cloning these methane genes are discussed below.

3.1. Electron supply

Hydrogenases have been purified from a range of methanogens (2,5,13,14) and characterized on the bases of the number and sizes of their sub-unit polypeptides, their physiological electron acceptor(s) and cofactors (2). Characterization of hydrogenases from *Methanobacterium thermoautotrophicum* resulted in the identification of the first biological nickel-based redox active site (13-16) and there is evidence that expression of these hydrogenase encoding genes may be regulated by nickel availability (15). Cloning hydrogenase encoding genes using antibodies and/or oligonucleotides as probes is now possible as purified hydrogenase enzymes are available from several methanogens (2). In collaboration with members of Dr C. Walsh's research group at MIT, members of the author's research group have already cloned and sequenced genes encoding subunits of the methylviologen (MV) reducing hydrogenase from *M. thermoautotrophicum* ΔH (2,13) and from a related strain, *M. thermoautotrophicum* Winter. *M. thermoautotrophicum* species contain a second hydrogenase which differs from the MV hydrogenase in that it reduces the methanogen-specific electron carrier known as Factor F₄₂₀. The available DNA sequences indicate that a subunit of the MV-reducing hydrogenase has amino acid sequences similar but not identical to amino acid sequences determined directly from a subunit of the F₄₂₀ reducing hydrogenase (C.T. Walsh, personal communication, 1987). It would appear therefore that these two hydrogenases, in *M. thermoautotrophicum*, have evolved from a common ancestor.

Formate dehydrogenases, purified from *Methanobacterium formicicum* (17,18) and *Methanococcus vannielii* (19), also reduce Factor F_{420} . The genes (*fdhA*, *fdhB*) encoding the two subunits of formate dehydrogenase in *M. formicicum* have been cloned and sequenced (18). These two genes overlap by one base pair, appear to be organized as an operon, and have been expressed in *E. coli*. Their expression in *M. formicicum* may be regulated competitively by molybdenum and tungsten availabilities (J.G. Ferry, personal communication, 1987). There is significant conservation of the amino acid sequences of the *fdhA* gene of *M. formicicum* and the *fdhF* gene of *E. coli* (20). However, where the *E. coli* gene has an in-frame TGA codon, apparently translated by a selenocysteinyl-tRNA, the methanogen *fdhA* gene has a normal cysteine-encoding TGC codon. The mode of incorporation of selenium into hydrogenases and formate dehydrogenases and the regulation of expression of hydrogenase and formate dehydrogenase encoding genes by metal ions are currently exciting topics of research.

Methanosarcina sp., growing on acetate, obtain reducing equivalents for use in methanogenesis by oxidation of carbon monoxide to carbon dioxide; a reaction catalysed by an enzyme known as CO-dehydrogenase (4,21,22). This enzyme, purified from *M. barkeri*, has two subunits (M_r of 92 000 and 18 000) whereas CO-dehydrogenase prepared from *M. thermophila* contains five subunits (M_r values of 89 000, 71 000, 60 000, 58 000 and 19 000 (22)) indicating that the latter enzyme may be a multifunctional complex. CO-dehydrogenase activity is several-fold higher in acetate-grown cells of *Methanosarcina* sp. than in methanol-grown cells, suggesting that these alternative substrates for methanogenesis regulate the expression of CO-dehydrogenase encoding genes in *Methanosarcina* (21,22). There are very few documented examples of metabolic regulation of enzyme levels/activities in methanogens so that cloning the CO-dehydrogenase encoding genes would be a very valuable first step towards determining a mechanism of regulation of gene expression in a *Methanosarcina* species.

Hydrogenases, formate dehydrogenases and CO-dehydrogenase are used by methanogens to obtain electrons (reducing equivalents) from externally supplied substrates. Enzymes that catalyse the subsequent intermediate steps, *in vivo*, by which these electrons are transferred to carbon reduction reactions, and the nature and sequence of the electron carriers employed have yet to be clearly delineated. Methanogenesis can, however, be obtained *in vitro* using reductant supplied from an artificial electron donor, such as dithiothreitol (23). Methanogenesis may therefore also be possible *in vivo* using electrons donated by reduced cofactors other than those normally used in methanogenic cells.

3.2. C1-substrate metabolism

Figure 1 shows, in outline, a circular pathway by which carbon dioxide is reduced in seven steps to methane. Enzyme preparations that catalyse steps 2,3,4,6 and 7

Table 1. Enzymes that catalyse steps in the reduction of carbon dioxide to methane (2,3).

Reaction step ^a	Enzyme	Subunits ^b (kDa)	Cofactors	References
1.	Formyl-methanofuran synthetase	ND	methanofuran	—
2.	Formyl methanofuran-tetrahydromethanopterin formyl transferase	4 × 41	methanopterin	27
3.	Methenyl tetrahydromethanopterin cyclohydrolase	2 × 41	methanopterin	25
4.	Methylene tetrahydromethanopterin F ₄₂₀ oxidoreductase	NR	methanopterin F ₄₂₀	24
5.	Methylene tetrahydromethanopterin reductase	ND	methanopterin	—
6.	Methyl tetrahydromethanopterin coenzyme M methyl transferase	NR	methanopterin coenzyme M corrinoid	26
7.	Methyl coenzyme M reductase ^c	61 46 30	coenzyme M component B F ₄₃₀ , B ₁₂ FAD, ATP, MG ²⁺	2,3

^aReaction steps are designated numerically as in Figure 1.

^bND, not demonstrated *in vitro*; specific details of these reactions and the enzymes involved remain to be determined. NR, subunits not reported; however, the enzymatic activity has been demonstrated *in vitro* (24,26).

^cThe subunits listed are for component C of methyl reductase of *M. vannielii* (29). There are three additional proteinaceous components A1, A2 and A3 needed *in vitro* for methyl reductase activity (2,3). A1 appears to be a hydrogenase (39); A2 has been purified to homogeneity (*M_r* of 59 K) and may be involved in ATP activation (40); A3 function is unknown.

in vitro have been isolated (23–27; Table 1). It should therefore be possible, using antibodies and/or oligonucleotides as probes, to isolate from recombinant gene banks, genes which encode these enzymes. The genes (*mcr*) that encode the subunits of component C of the enzyme, methyl coenzyme M reductase, a component of the enzyme complex that catalyses step 7 in Figure 1, have been cloned using this approach (28,29). Surprisingly, there are five *mcr* genes, *mcrBDCGA*, in *Methanococcus vannielii*. The β , γ and α polypeptide subunits of the purified enzyme are encoded by *mcrB*, *mcrG* and *mcrA* respectively. The function(s) of the *mcrD* and *mcrC* gene products are unknown (29).

The reaction catalysed by methyl reductase (step 7 in Figure 1) is common to all methanogenic pathways. The cycle shown in Figure 1 depicts carbon dioxide reduction to methane; however, methyl coenzyme M (CH₃—S—CoM) is also an intermediate in the reduction to methane of methyl groups derived from methanol, methylamines and acetate in *Methanosarcina* species (2–4). There are substrate-specific, substrate-inducible methyl transferase enzymes in *Methanosarcina* which transfer methyl groups from methanol (30,31) or methylamines (32) to HS—CoM

to form $\text{CH}_3\text{—S—CoM}$. These methyl transferase enzymes have been purified (30–32) so it should be possible to clone the encoding genes and to study how their expression is regulated by substrate availability. As discussed earlier, it seems possible that viable mutants could also be isolated with mutations in one or more of these methyl transferase encoding genes. Cloned methyl transferase genes could easily be mutated *in vitro*. A transformation system for *Methanosarcina* is needed, however, before such mutated genes could be re-introduced into viable cells. Enzyme(s) that catalyse the transfer of the CH_3 moiety of acetate to HS—CoM must also exist but have yet to be characterized (4). As stated above, CO-dehydrogenase appears to play a role in the pathway of synthesis of methane via $\text{CH}_3\text{—S—CoM}$ from acetate (4,21,22) and it would be very interesting to determine whether mutations in CO-dehydrogenase prevent methanogenesis from acetate in *Methanosarcina*.

3.3. Cofactors

Methanogens employ at least six unusual cofactors in methanogenesis (Figure 1; Table 2). Three of these—methanofuran, methanopterin and coenzyme M—act as one-carbon carriers at sequential steps in the reduction of carbon dioxide to methane. Factor F_{420} is a two-electron carrier which donates electrons at step 4 (Figure 1) and also functions as a cofactor in several other oxido-reduction reactions in methanogens (1–3). Factor F_{430} and component B play essential but, as yet, unclear roles in step 7 (Figure 1). Until alternative cofactors are shown to function in methanogenesis, the genes encoding the enzymes that catalyse the biosynthesis of these six cofactors must also be considered essential 'methane genes'. There is, unfortunately, only very limited knowledge of the reactions which result in the syntheses of these cofactors. Factor F_{420} , a diazoflavin, appears to be synthesized via a pathway similar to that leading to flavins and pterins in eubacteria (33). Isotopic tracer studies suggest that methanopterin biosynthesis is similar to folate biosynthesis (34,35). Factor F_{430} , a nickel-containing tetrapyrrole, is synthesized via a pathway common to the biosyntheses of all porphyrinoid cofactors, including the chlorophylls, haems, sirohaems and cobalamines (23,36,37) but which must also have additional enzymes specific for the terminal steps in F_{430} biosynthesis which incorporate a nickel atom into the cofactor. Coenzyme M, a relatively simple organic compound (2-mercaptoethanesulphonic acid), is synthesized from acetate and sulphite via 3-sulphopyruvate and sulphacetaldehyde (38). The biosynthetic pathways leading to component B (7-mercaptoheptanoylthreonine phosphate) and methanofuran have not been determined.

4. Conclusions

This paper started with a question; can an answer be provided? Certainly the biochemical steps directly involved in carbon reduction are catalysed by enzymes,

such as methyl coenzyme M reductase and methyl transferases, which are encoded by genes justifiably designated as 'methane genes'. If, however, one wishes to define methane genes more broadly, for instance as those which must be transferred and expressed in *E. coli* to make *E. coli* into a methanogen, then the list of methane genes would have to include genes for electron supply and cofactor biosyntheses. Making *E. coli* into a methanogen would seem, however, not only technically very difficult but also pointless. A more tractable and productive goal might be to introduce genes, such as those required to degrade aromatic and/or toxic compounds, into methanogens. Species that could generate methane directly from a wide range of substrates, particularly substrates commonly found in anaerobic biomass and waste digesters, many of which are man-made toxic pollutants, could be very valuable.

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